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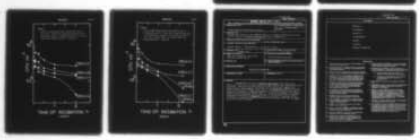

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MENINGOCOCCAL CONTROL IN THE CANADIAN FORCES III. HANDLING PROCEDURES TO ENSURE MAXIMAL RECOVERY OF *Neisseriae meningitidis* FROM AIR SAMPLES (U)

by

B.E. Holbein, M.R. Spence and L.A. White

Technical Program 16 - Operational Medicine

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ABSTRACT

Representative strains of *Neisseria meningitidis* were found to be sensitive to holding in Trypticase Soy Broth (TSB) at 20° C, after aerosolization and collection. The viability losses observed during holding were suggestive of aerosolization-induced damage. The strains of *N. meningitidis* examined were also found to be sensitive to freezing and thawing. Of several cryoprotective agents examined, only dimethyl sulphoxide (DMSO) at concentrations between 5 and 10% was found to provide protection during freezing. It was concluded that air samples suspected of containing *N. meningitidis* should be plated for assessment within 10 minutes, or treated with DMSO to a concentration between 5 and 10%, and frozen on dry ice, within 10 minutes. (U)

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INTRODUCTION

Air sampling for *Neisseria meningitidis* constitutes an important part of the overall meningococcal control program being conducted at the Canadian Forces Bases (CFB) St. Jean and Cornwallis (2). Air samples are collected at these bases both on a routine basis, when there are no overt cases of meningitis, and on an intensive basis, when a case occurs.

Air samples are collected by using the Defence Research Establishment Suffield (DRES) - modified large-volume air sampler, which collects the particulates from 1000 litres of air into 1 ml of collection fluid per minute (4, 9 - 10). The usual collection fluid employed is Trypticase Soy Broth (TSB). Samples are then assessed for the presence of *N. meningitidis*, by culturing on media with or without selective antibiotic supplements. Delay in assessment of samples is common, particularly when samples are collected at locations remote from the base laboratory facilities (e.g. in barrack blocks), or when samples are shipped to DRES for assessment (2,7). The effect of this holding time (i.e. time between sample collection

and assessment) on the viability of *N. meningitidis* had yet to be determined, under controlled conditions.

Samples to be shipped to DRES from CFB's are placed in dry ice and forwarded in appropriate containers to ensure that the samples remain frozen during transit (7). Viable cells are easily recovered from such samples when they contain greater than 10^8 colony forming units (CFU) per ml (L.A. White, unpublished results). However, there is some doubt that meningococcal cells can survive freezing and thawing, without the aid of a cryoprotectant, when samples contain less than 20 CFU per ml. The latter situation (i.e. low cell numbers in samples) is common when samples are collected from naturally occurring aerosols.

The present study was initiated to assess the effects of holding and freezing on the viability of *N. meningitidis* after collection from aerosol. Various agents were also evaluated for their possible cryoprotective action during freezing. Cells collected from artificially generated aerosols were employed, rather than cells from natural aerosols, so that controlled conditions could be achieved.

MATERIALS AND METHODS

The four *N. meningitidis* strains employed were originally isolated at CFB Cornwallis, and included two serogroup B strains (DRES - 01 and DRES - 17) and two serogroup C strains (DRES - 04 and DRES - 06). The strain designations are those assigned at DRES. Both strains DRES - 01 and DRES - 06 were isolated from cases of overt meningitis. Strain DRES - 17 was isolated from the air, and strain DRES - 04 was isolated from the nasopharynx of an apparently healthy carrier. Cell cultures were grown in TSB to a density of ca 10^8 CFU per ml, as described elsewhere(8).

Cells were aerosolized from a Collison nebulizer (3), directed into a rotating drum apparatus and held at a relative humidity of 45%, in the manner described elsewhere (8). Samples were collected from the drum into TSB using AGI-15 impingers, 11 minutes after filling of the drum had ceased (impinger count, ca 10^4 CFU ml^{-1}). The effects of holding in collecting fluid on the viability of

N. meningitidis were determined by incubating samples at 20° C. Samples were withdrawn at intervals for up to 2h, and viable cell numbers were determined. Controls consisted of non-aerosolized cells held and sampled under identical conditions.

Samples (2ml) were frozen in glass, screw-cap test tubes (13 x 75 mm), by three methods; 1) in a -60°C ultra-low chest freezer (Revco Ind., Deerfield, Mich.); 2) on solid dry ice, and 3) in a dry ice/ethanol bath. All frozen samples were held at -60°C for 7 days, then thawed rapidly under running, warm (45°C) water, prior to assessment. The various agents were tested for their cryoprotective capabilities by incorporating them directly into the TSB freezing menstruum. The following agents were used: glycerol (Fisher Scientific Co., Edmonton, Alberta); dimethyl sulphoxide (DMSO) (Fisher); skim milk (Difco Laboratories, Detroit, Mich.); sorbitol (Nutritional Biochemical Co., Cleveland, Ohio), and bovine serum albumin (BSA), fraction V (Nutritional Biochemical Co.).

Viability of *N. meningitidis* in all samples was determined by plating on Columbia agar (Grand Island Biological Co., (GIBCO), Grand Island, N.Y.), containing 4% sheep red blood cells, Iso Vitalex (BBL, Cockeysville, Ma.), and VCNT (GIBCO). Colonies arising from viable cells were counted after 18h growth at 35°C in an atmosphere containing 5% CO_2.

RESULTS

Effect of Holding in TSB On the viability of *N. meningitidis*

The four strains of *N. meningitidis* examined did not suffer detectable viability losses prior to aerosolization, during holding in TSB at 20°C for up to 2h (see Figure 1). Growth (in terms of increased viable numbers) was evidenced for all four strains under these conditions. This was not surprising, as TSB had initially been used to grow the cultures prior to experimentation. Aerosolized *N. meningitidis* behaved very differently during holding after collection. There was no evidence for growth, but rather, there were viability losses observed for all four strains (see Figure 2). Strain DRES - 04 appeared to be the most sensitive during holding.

Viability losses for the different strains during the 2h holding period were as follows: 56% for DRES - 01; 57% for DRES - 17; 75% for DRES - 06, and 89% DRES - 04 (data computed from Figure 2, and assuming no increased cell numbers from surviving fraction).

Effects of Freezing and Cryoprotective Agents On Viability

It became important to determine both the magnitude of any loss of viability suffered through a freezing/thawing process, and whether or not freezing samples on dry ice, as previously suggested (7), was the best method available for preserving the viability of *N. meningitidis*. Table 1 (control) summarizes the effects that the three freezing methods had on the subsequent recovery of aerosolized *N. meningitidis*. Freezing by any of the methods resulted in substantial losses in viability for all three strains examined. Recovery was as low as 26% in the case of strain DRES - 04, when frozen in a -60°C chest freezer, and as high as 77%, in the case of strain DRES - 06, when frozen on dry ice. The data suggested that freezing on dry ice, or in a dry ice-ethanol bath, was superior to freezing in a -60°C chest freezer.

Cryoprotective agents were examined, with the aim of selecting an agent capable of protecting *N. meningitidis* from the viability losses suffered during freezing and thawing. Table 1 summarizes the results obtained when the five cryoprotectants were incorporated into the freezing medium. Only DMSO appeared to provide a significant measure of protection from freeze/thaw effected viability losses. Glycerol and sorbitol appeared to have detrimental effects on recovery. BSA appeared to provide marginal protection in some cases. The effects that the various agents had on recovery were mirrored in experiments conducted with non-aerosolized cells (Holbein, Spence, and White, unpublished results). DMSO, at concentrations ranging from 5 to 10%, appeared to provide the same degree of protection for all four strains of *N. meningitidis* used in this study (results not shown).

It was necessary to determine whether or not DMSO would prove toxic to *N. meningitidis*, while present prior to freezing. Figure 3

shows the effects that 5% DMSO had on the viabilities of *N. meningitidis* strains, during holding at 20°C. Exposure of aerosolized cells of strains DRES - 17 or DRES - 01 to 5% DMSO for greater than 10 minutes resulted in marked drops in viabilities for both strains. DMSO toxicity appeared not to be a problem with any of the strains, if samples were frozen within 10 minutes after DMSO additions, and plated for assessment immediately after thawing.

DISCUSSION

The results of the present study have shown that artificially aerosolized *N. meningitidis* strains are sensitive to holding in TSB collection fluid at 20°C. The results suggested that damage to *N. meningitidis* strains, effected during aerosolization, or while in aerosol, was being expressed during holding in TSB. The rapid losses in viability during holding were consistent with the latter suggestion (see Figure 2). The nature and the extent of the damage remain obscure. We do have evidence that artificially aerosolized *N. meningitidis* become sensitive to certain antibiotics and become reduced in their ability to oxidize tetramethyl phenylendiamine (i.e. reduced "oxidase" activity); and have observed that certain strains, when grown and aerosolized in TSB (a proteinaceous medium), exhibit much reduced viability if collected in Neisseria Chemically Defined Medium (NCDM, a non-proteinaceous medium) (unpublished results of this laboratory). All of the available evidence has led us to speculate that one of the prime sites for aerosolization-induced damage may be the permeability barrier of the organism. It would appear reasonable to assume that naturally occurring aerosolized *N. meningitidis* would be damaged in a similar manner, thus necessitating special measures to ensure their reliable detection in samples collected from the air. We suggest, based on our evidence, that samples collected from the air should be plated within 10 minutes, or frozen in the presence of 5 - 10% DMSO on dry ice, for later assessment.

Freezing of samples in an ultra-low freezer (-60°C), or in a domestic deep freeze (-20°C) is contraindicated, as recoveries were low in the former (see Table 1), and very low in the latter

(L.A. White, unpublished). Samples can be stored at -60°C after they are frozen, however. The effects that the different freezing methods had on recovery were undoubtedly related to the cooling rate during freezing, as all samples were stored at -60°C prior to assessment. The exact relationship between survival and cooling rate was not established. However, other workers, using controlled rates of cooling, have shown that bacterial survival, in general, is related to the cooling rate (1). It has also been shown that rapid rewarming from the frozen state is necessary for high survival rates (1).

Of the cryoprotective agents examined, only DMSO was found to protect *N. meningitidis* during freezing. Recoveries with 10% DMSO were as high as 82%, when samples were frozen on dry ice. It is important to note that 5% DMSO did cause a reduction in viable counts, if present for longer than 10 minutes prior to freezing. Samples frozen within 10 minutes and plated immediately after thawing were not affected by DMSO. It would appear reasonable to assume that 10% DMSO would prove to be of higher toxicity, necessitating the rapid freezing of samples after DMSO additions.

Glycerol, a commonly used cryoprotectant (5), was found not to protect *N. meningitidis*, and we do not recommend its usage with *N. meningitidis*, even though it has been used for a related organism, *N. gonorrhoeae* (6).

CONCLUSIONS

Representative strains of *N. meningitidis* have been found to be sensitive to holding after aerosolization and collection, in a manner implicating aerosolization - affected damage. *N. meningitidis* was also found to be sensitive to freezing and thawing, with best recoveries obtained when samples were frozen on dry ice in the presence of 5 - 10% DMSO. We conclude that air samples suspected of containing *N. meningitidis* should be plated for assessment immediately, or treated with DMSO to a concentration between 5 and 10%, and frozen on dry ice, within 10 minutes. Frozen samples should be thawed rapidly under running warm water, and plated for assessment immediately.

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Table 1

Effects of freezing and cryoprotectants on recovery of *N. meningitidis*. *N. meningitidis* strains were grown, aerosolized, and collected in TSB. Samples were frozen in TSB with or without the presence of cryoprotective agents using three freezing methods.

Table 1

Recovery %[1]

Treatment	DRES - 06			DRES - 04			DRES - 17		
	A	B	C	A	B	C	A	B	C
TSB Control	29	77	36	26	55	32	46	67	68
TSB & 10% DMSO	48	82	69	76	68	69	51	80	77
TSB & 20% Glycerol	36	14	40	4	1	12	21	14	19
TSB & 20% Sorbitol	17	21	13	1	< 1	1	1	4	8
TSB & 2% BSA	36	52	60	41	59	28	66	45	66
TSB & 2% Skim Milk	36	32	27	15	28	39	27	58	41

1 Recovery expressed as % of unfrozen controls.

A. Freezing in a -60°C chest freezer.

B. Freezing on dry ice.

C. Freezing in a dry ice/ethanol bath.

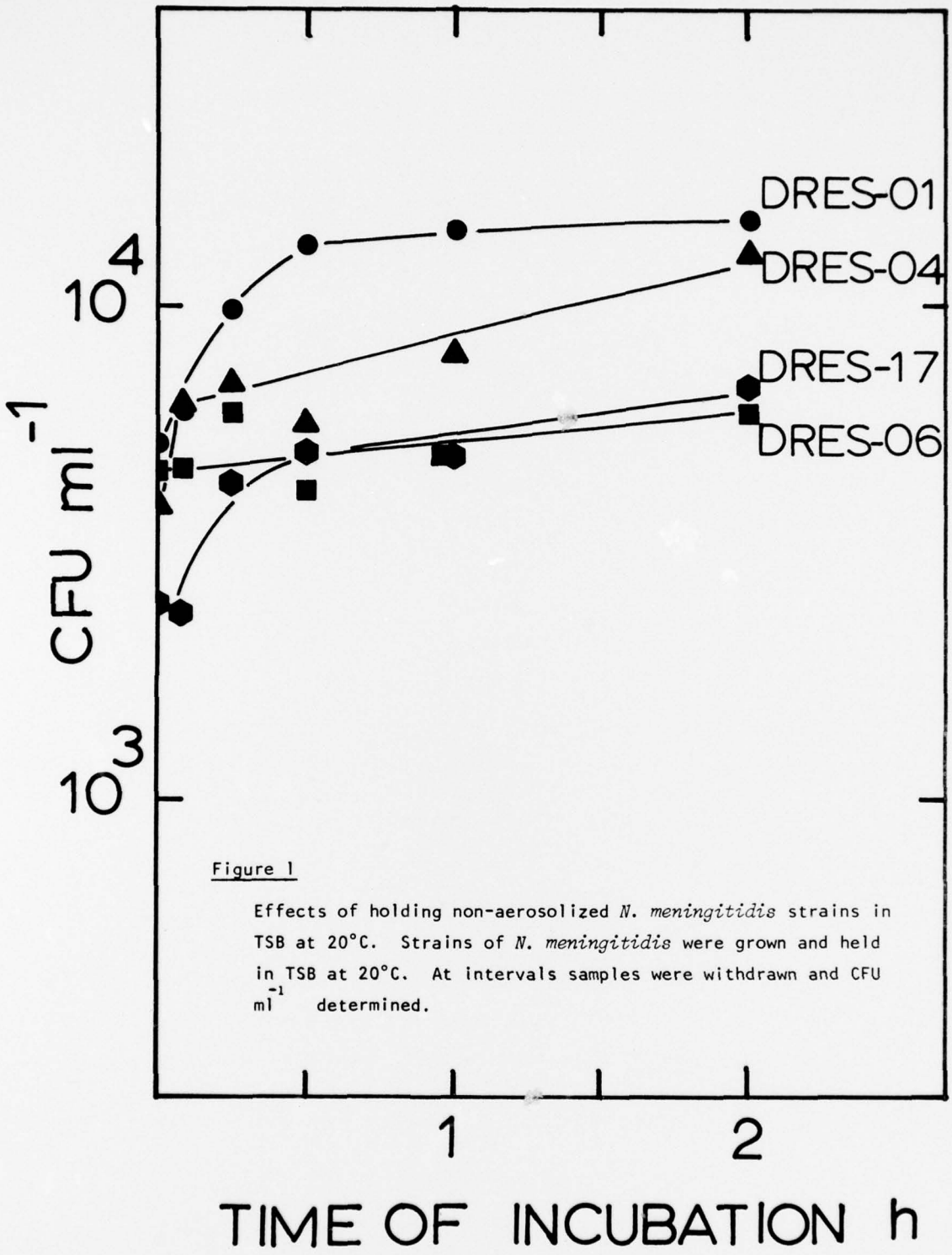


Figure 1

Effects of holding non-aerosolized *N. meningitidis* strains in TSB at 20°C. Strains of *N. meningitidis* were grown and held in TSB at 20°C. At intervals samples were withdrawn and CFU ml^{-1} determined.

Figure 2

Effects of holding aerosolized *N. meningitidis* strains in TSB at 20°C. Strains of *N. meningitidis* were grown in TSB, aerosolized, collected in TSB, and held at 20°C. At intervals samples were withdrawn and CFU ml^{-1} determined.

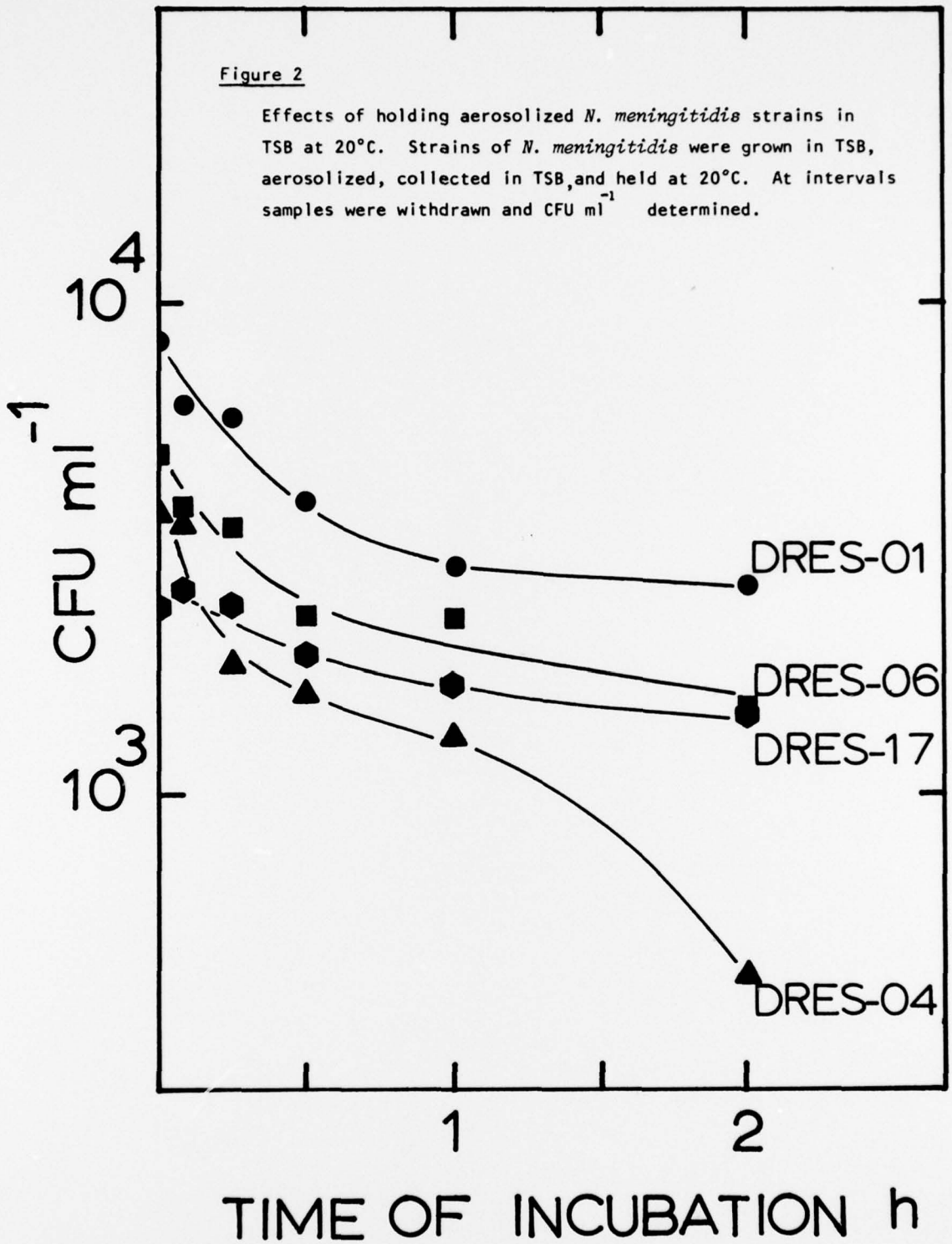
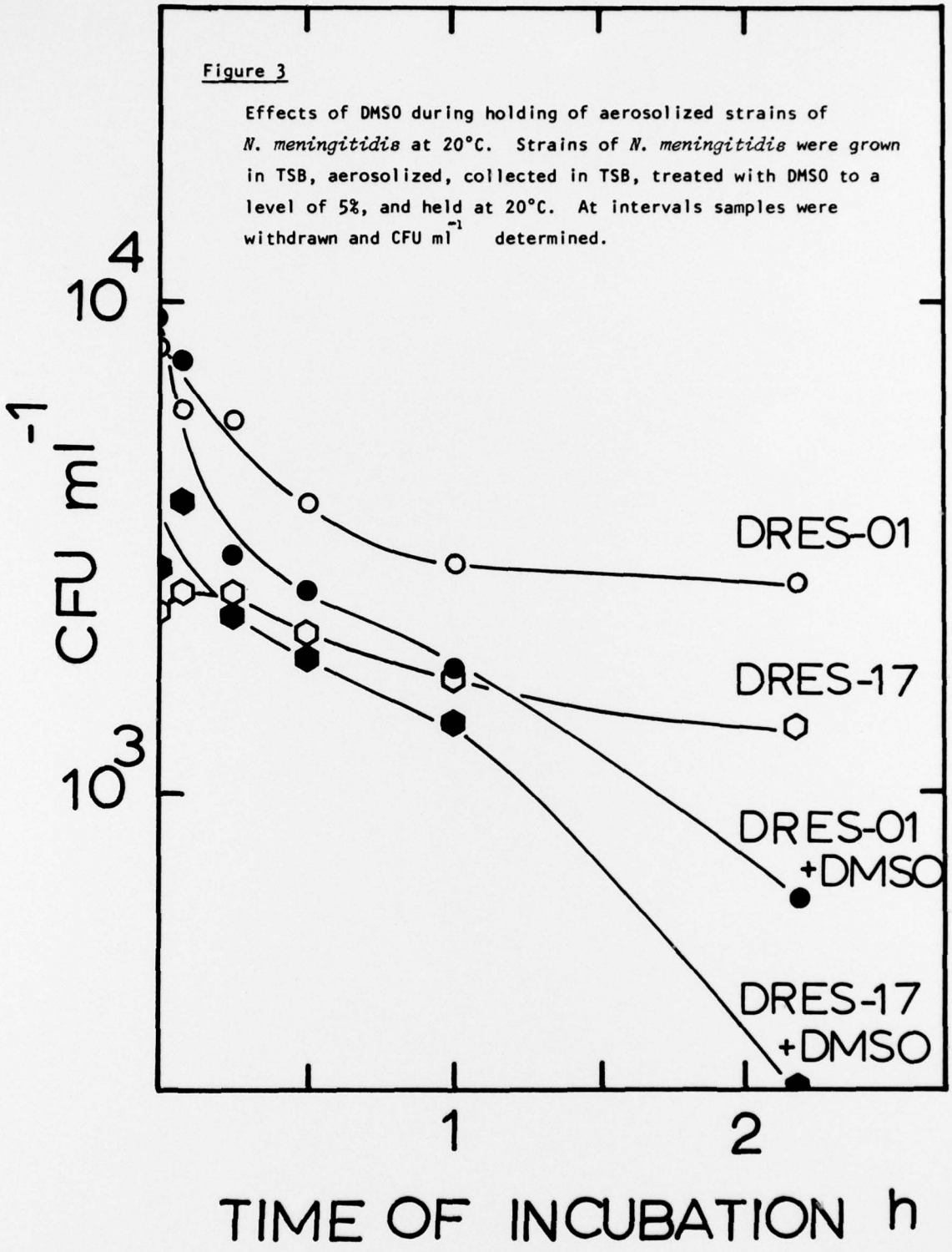


Figure 3

Effects of DMSO during holding of aerosolized strains of *N. meningitidis* at 20°C. Strains of *N. meningitidis* were grown in TSB, aerosolized, collected in TSB, treated with DMSO to a level of 5%, and held at 20°C. At intervals samples were withdrawn and CFU ml^{-1} determined.



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